Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 2-16, 18-29, 61, 62, 64-85 are pending in the application, with claims 2 and 6 being the independent claims. Claim 63 is sought to be cancelled without prejudice to or disclaimer of the subject matter therein. New claims 80-85 are sought to be added. Support for new claims 80-85 may be found *inter alia*, on page 6, lines 8-26; page 26, lines 3-27; and Figure 1. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Objections under 37 C.F.R. § 1.75(c)

Claims 8 and 9 were objected to under 37 C.F.R. § 1.75(c) as allegedly being of improper dependent form for failing to further limit the subject matter of a previous claim. Applicants respectfully traverse.

Amended claim 8, which depends on claim 6, is drawn to a host cell which expresses at least one of the listed activities. Claim 6 is a method of transforming a *Corynebacterium* species host cell. There is however, no requirement for the host cell of claim 6 to possess the activities listed in claim 8, *i.e.* a native *Corynebacterium* cell. The host cell may be a mutant cell in which one or more of the listed activities has been deleted. The existence of mutant bacteria and methods to make such bacteria are well known in the art. Therefore,

Applicants respectfully submit that the objection to claims 8 and 9 is improper and request its withdrawal.

Claim 63 was also objected to under 37 C.F.R. § 1.75(c) as allegedly being of improper dependent form for failing to further limit the subject matter of a previous claim. Applicants have canceled claim 63. Therefore, the objection has been rendered moot and Applicants respectfully request that it be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph

The previous rejection of claims 8 and 9 under 35 U.S.C. § 112, first paragraph, written description, was maintained. Applicants respectfully traverse.

Applicants respectfully disagree with the Examiner's assertion that a description of the common characteristics of each genus, particularly a correlation between the structure and function of the genes described is necessary to identify other member of each genus in structural and functional terms. Applicants assert that since assays involving lysine biosynthesis were well known in the art and also described in the specification, there is no requirement to have identified the structure of each and every member of the genus to fulfill the requirement of written description because techniques useful in the identification of other members of the genus was known in the art.

In support of this assertion, Applicants enclose a copy of Tokunaga *et al.* (*J. Protein Chem. 18*:837-844 (1999)) ("Tokunaga") (Exhibit A). In Tokunaga, representative of the state of the art at the time of filing, the authors used the characteristic of growth at elevated temperatures to identify a protein involved in said growth in *Halobacterium cutirubrum*. The authors further identified amino-terminal sequences of internal sequences of cryptic

peptides and found that the sequences coincided with the deduced nucleotide sequence of the dnaK gene from *H. cutirubrum*. Therefore, the authors identified a member of the genus involved in growth at elevated temperatures using assays known in the art, and further identified the structure of said member using techniques known in the art at the time of filing.

For the claimed invention, Applicants describe polypeptides which are involved in lysine biosynthesis (Figure 1). Assays to identify other members of each genus described in Figure 1 could be used involving, *inter alia*, measurement of the described enzymatic activities. Using the disclosure and methods known in the art, as exemplified by Tokunaga, one of ordinary skill would recognize that the inventors had possession of the claimed invention at the time of filing. Therefore, Applicants respectfully request that the rejection be withdrawn.

Claims 68-72 were also rejected under 35 U.S.C. § 112, first paragraph, written description, as allegedly failing to comply with the written description requirement. Applicants respectfully traverse the rejection for the reasons stated above, and request that the rejection be withdrawn.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 6-15 and 67-72 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Applicants have amended claims 6 and 67 as suggested by the Examiner. Therefore, Applicants believe that the rejection has been rendered moot, and respectfully request that it be withdrawn.

Claims 8-9 and 68-72 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Applicants have amended claim 8 to recite the phrase "possess at least one of the following activities:". In addition, as mentioned above, Applicants respectfully assert that though native *Corynebacterium* contains the activities listed in claim 8, claim 6, from which claim 8 depends, does not limit the *Corynebacterium* host cell to native cells. Therefore, Applicants respectfully request that the rejection be reconsidered and withdrawn.

Claims 68-72 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Applicants have amended claim 8 as suggested by the Examiner. Therefore, Applicants believe that the rejection has been rendered moot, and respectfully request that it be withdrawn.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Michele A. Cimbala
Attorney for Applicants
Registration No. 33,851

Date: 8/22/03

1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

EXHIBIT A

Journal of Protein Chemistry, Vol. 18, No. 8, 1999

Identification and Partial Purification of DnaK Homologue from Extremely Halophilic Archaebacteria, *Halobacterium cutirubrum*

Hiroko Tokunaga,[1] Shinichi Hara,[1,3] Tsutomu Arakawa,[1,4] Matsujiro Ishibashi,[1] Radhey S. Gupta,[2] and Masao Tokunaga[1,5]

Received December 1, 1999

The levels of synthesis of six proteins were increased at elevated growth temperature of the extremely halophilic archaebacterium *Halobacterium cutirubrum*. One of these proteins, with an apparent molecular mass of 97 kDa on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), bound to an ATP-agarose column in the presence of 4 M NaCl, but not in the absence of salt, indicating that this protein retained its ATP-binding activity only at high salt concentration. The NH_2-terminal sequence of this protein and the internal sequences of the tryptic peptides covering 1/3 of the total number of residues coincided with that deduced from the nucleotide sequence of the *dnaK* gene isolated from *H. cutirubrum*. The results strongly suggest that this apparent 97-kDa protein is the gene product of *dnaK*, although the molecular mass calculated from the nucleotide sequence is only 68,495, much smaller than the value of this protein determined by SDS–PAGE. Ferguson plot analysis indicated that this protein showed anomalous mobility on SDS–PAGE. We have purified DnaK homologue to greater than 90% homogeneity with stepwise elution from an ATP-agarose column.

KEY WORDS: Archaebacteria; ATP-agarose column; DnaK protein; halophiles; *Halobacterium cutirubrum;* Hsp70 homologue.

1. INTRODUCTION

Marked changes in the immediate environment result in a variety of responses in living organisms. Many investigators have shown that the heat shock response is a common reaction in all forms of life, not only in eukaryotes and eubacteria, but also in archaebacteria. Major heat shock proteins (Hsps)[6] DnaK and GroESL in *Escherichia coli* have been extensively studied (Hartl, 1996; Bukau and Horwich, 1998), and their three-dimensional structures as well as biochemical properties have been well characterized (Braig *et al.*, 1994; Zhu *et al.*, 1996; Xu *et al.*, 1997).

However, little information is available regarding the homologues of major Hsps from the extremely halophilic archaebacteria. Daniels *et al.* (1984) first reported the protein profiles of the archaebacteria genus *Halobacterium* in response to heat shock, which showed that the levels of synthesis of a limited number of proteins which fell into three narrow molecular weight ranges were increased when cells were treated at elevated temperature. Kuo *et al.* (1997) reported that two heat-responsive *cct* genes from Archaeon

[1] Laboratory of Applied and Molecular Microbiology, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan.
[2] Department of Biochemistry, McMaster University, Hamilton, Ontario L8N3Z5, Canada.
[3] Present address: Amgen Inc., Amgen Center, Thousand Oaks, California 91320-1789.
[4] Present address: Alliance Protein Laboratories, Thousand Oaks, California 91360.
[5] To whom correspondence should be addressed; E-mail: tokunaga@chem.agri.kagoshima-u.ac.jp.

[6] Abbreviations: Hsp, heat shock protein; IPTG, isopropyl β-D-thiogalactopyranoside; TCA, trichloroacetic acid; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis.

0277-8033/99/1100-0837$16.00/0

Haloferax volcanii were induced after heat shock. The Hsp70 genes (*dnaK* homologues) from halophilic archaebacteria were cloned from *Halobacterium marismortui* (Gupta and Singh, 1992) and *H. cutirubrum* (Gupta and Singh, 1994), and their sequences were discussed from the viewpoint of the evolutionary relationships among archaebacteria, eubacteria, and eukaryotic cells. However, DnaK protein in extremely halophilic archaebacteria has not yet been identified.

Here, we describe the identification, partial purification, and biochemical characterization of the DnaK homologue from *H. cutirubrum*.

2. MATERIALS AND METHODS

2.1. Bacterial Strain and Medium

The extremely halophilic bacterium *H. cutirubrum* was obtained from ATCC (Number 33170). It was maintained on Sehgal and Gibbons complex (SGC) agar medium (1% yeast extract, 0.3% sodium citrate, 0.2% KCl, 2% $MgSO_4$, 0.0023% $FeCl_2$, 0.75% vitamin free casamino acids, and 2% agar, pH 7.0; Sehgal and Gibbons, 1960) containing 3 M NaCl. Cells were usually grown at 37°C in SGC liquid medium containing 4 M NaCl, and grown at 25, 30, 37, 42, or 52°C to late exponential phase to examine the expression of heat-inducible proteins. *E. coli* BL21 (DE3) was used as host of pET3a expression system. Luria–Bertani broth (LB broth, 1% bacto-tryptone, 0.5% bacto-yeast extract, 0.5% NaCl, pH 7.0) was used for *E. coli* BL21 (DE3).

2.2. Preparation of Cell-Free Extracts

Cells grown in 5-ml cultures were suspended in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.5, 25 mM KCl, and 2 mM $MgCl_2$ (buffer A) containing 4 M NaCl and were frozen at −80°C with 0.5 ml of glass beads (0.5 mm in diameter). The frozen samples were mixed vigorously for 5 min to disrupt cells, and then frozen again. This freeze–thaw treatment was repeated at least three times. To examine the salt requirement of proteins, cells were also suspended in buffer A containing 0 and 2 M NaCl, respectively, and were disrupted as described above.

2.3. Chromosomal DNA Preparation

Cells grown in 200 ml of culture were washed with 50 ml of 3.5 M NaCl containing 0.2 mM $MgSO_4$ and suspended in 20 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. Following addition of 20 ml of phenol equilibrated with 0.5 M Tris buffer (pH 8.0) and incubation for 30 min at 37°C, aqueous phase was obtained. Extraction with phenol was repeated two times. NaCl was added to the aqueous phase to give a final concentration of 0.5 M, and 20 ml of cold ethanol was added. DNA was spooled on the glass rod and purified according to Mevarech *et al.* (1989).

2.4. Construction of Plasmid

The region encoding the *H. cutirubrum danK* gene was amplified by PCR using forward (5′-GGGCATATGGCGAGCGAGAAGATTCTGGGA-3′) and backward (5′-GGGGGATCCTTACTCCTCGTCGACGTCCTC-3′) primers.

The amplified fragment was digested with *Bam*HI and then ligated into *Sma*I/*Bam*HI-digested pUC19 to constract plasmid pUC19HcK. The *H. cutirubrum dnaK* gene-encoding region of pUC19HcK was digested with *Nde*I and *Bam*HI and ligated into the *Nde*I/*Bam*HI-digested pET3a. The constructed plasmid pET3HcK was used for the expression of *H. cutirubrum* DnaK homologue.

2.5. Expression of *H. cutirubrum* DnaK Homologue

E. coli BL21(DE3) harboring plasmid pET3a or pET3HcK was grown in LB broth containing 100 μg ampicillin and 0.4% glucose at 37°C. After 2.5 hr of incubation, the expression of DnaK homologue was induced by the addition of IPTG to give a final concentration of 0.2 mM and cultivation was continued for an additional 2.5 hr. Cells were harvested and disrupted by sonic oscillation. The cell lysates were centrifuged at 12,000 × *g* for 15 min and the soluble fractions were run on SDS–PAGE.

2.6. ATP-Agarose Affinity Column Chromatography

The cell homogenate was centrifuged at 12,000 × *g* for 30 min. The clear lysates from the disrupted cells in buffer A containing 4 M NaCl were applied to ATP-agarose resin (0.6 ml in BioRad polyprep column) equilibrated with the same buffer. After washing the ATP agarose column with two bed volumes of buffer A containing 4 M NaCl, the bound proteins were eluted with three bed volumes of the same buffer solution containing 4 M NaCl and 3 mM ATP. To the

eluted fractions was added TCA to a final concentration of 10%. The TCA-precipitated proteins were washed with acetone twice to remove TCA and then subjected to SDS–PAGE. To study the effects of salt concentration on the results of ATP-agarose column chromatography, buffer A containing 0 or 2 M NaCl was also used for cell disruption or equilibration, washing, and elution of the ATP-agarose column.

2.7. NH_2-Terminal and Internal Amino Acid Sequence Analyses

The proteins eluted from the ATP-agarose column were separated by SDS–PAGE and transferred to ProBlott membrane by electroblotting at 200 mA for 1 hr in 3-cyclohexylamino-1-propanesulfonic acid buffer, pH 10.5. The membrane was stained with 0.2% Coomassie brilliant blue in 40% methanol. After destaining with 50% methanol, the main band migrating at 97 kDa was cut out carefully with a razor blade and was subjected to NH_2-terminal amino acid sequence analysis (Applied Biosystems, Model 477A). For internal sequences, the protein on excised ProBlott membrane was digested with trypsin, and the resultant peptides were separated with a C_{18} reversed-phase HPLC column as described elsewhere (Haniu *et al.*, 1996).

2.8. Others

SDS–PAGE was performed by the method of Laemmli (1970). Standard Laemmli sample buffer containing 5% β-mercaptoethanol was used and samples were heated at 95°C for 5 min. Amount of protein was measured as described by Lowry *et al.* (1951). The reagents used were of the highest grade commercially available.

3. RESULTS

3.1. Determination of Heat-Inducible Proteins and Their Affinities to the ATP-Agarose Column

To examine the highest growth temperature of *H. cutirubrum*, it was grown at various temperatures up to 55°C in SGC liquid medium containing 4 M NaCl. We found that *H. cutirubrum* grew at temperatures up to 52°C. We initially studied the heat-inducible synthesis of proteins in *H. cutirubrum*. Cells grown at 24, 30, 37, 42, and 52°C for 3–6 days to stationary phase (growth rate was dependent on temperature) were disrupted in buffer A containing 4 M NaCl as described in Materials and Methods, and heat-induced proteins in the cell homogenates were determined by SDS–PAGE. As shown in Fig. 1, heat-inducible proteins with molecular masses of around 90 kDa (band II and band III), 55 kDa (band IV), 46 kDa (band V), and 31 kDa (band VI) were observed.

Since major heat shock proteins Hsp70s and Hsp60s possessed ATPase activity and bound to the ATP-agarose column, and proteins from halophilic archaebacteria usually required high concentrations of salt for their structural and functional integrity, we examined the salt requirement of the identified proteins. The cells grown at 42°C were disrupted in buffer A containing 0, 2, or 4 M NaCl. Respective cell-free extracts were applied to ATP-agarose columns equilibrated with buffer A containing 0, 2, or 4 M NaCl. After washing the column, bound proteins were eluted with buffer A containing 3 mM ATP and 0, 2, or 4 M NaCl, respectively, and eluted proteins were analyzed by SDS–PAGE. A protein with a molecular mass of

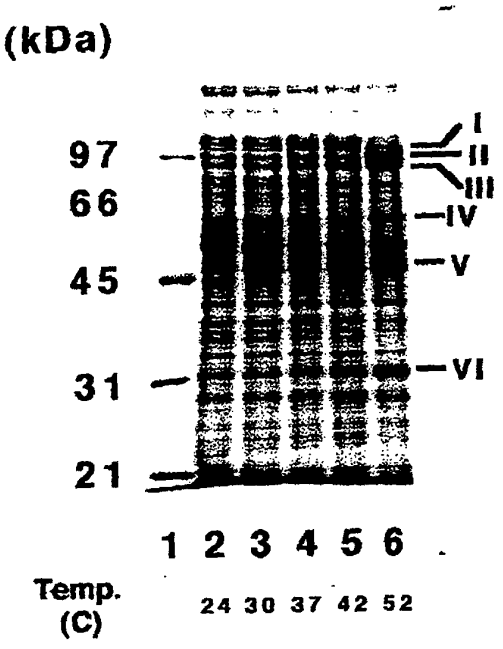

Fig. 1. SDS–polyacrylamide gel electrophoretogram of cell-free extract from *Halobacetrium cutirubrum*. The cells were grown at different temperatures. *Lane 2*, 24°C; *lane 3*, 30°C; *lane 4*, 37°C; *lane 5*, 42°C; *lane 6*, 52°C. In each lane, 20 μg of protein was loaded and the gel was stained with Coomassie brilliant blue. Low-molecular-weight standards (BioRad) were applied in *lane 1*. The bands from I to VI show heat-inducible proteins. The heat-inducible property of band I protein is discussed in the text.

around 97 kDa (97-kDa protein; arrowhead in Fig. 2) exhibited the strongest dependence on the salt concentration, since this protein bound to the ATP-agarose column in the presence of 4 M NaCl, while less or almost no 97-kDa protein bound to the ATP-agarose column in the presence of 2 or 0 M NaCl, respectively. These observations indicated that the 97-kDa protein required salt for binding to the ATP-agarose column. In contrast, DnaK and GroEL in nonhalophilic *E. coli* and their homologues in moderately halophilic *Acinetobacter* and *Pseudomonas* species bound to the ATP-agarose column in the absence of NaCl (Tokunaga *et al.*, 1997*a,b*). Careful inspection of the SDS–polyacrylamide gel electrophoretogram (6.5% acrylamide concentration) showed that this 97-kDa protein which bound to the ATP-agarose column was not identical to the major heat-inducible proteins around 90 kDa (neither band II nor band III in Fig. 1), but corresponded to the more upper band of band II (we called this band I). To clarify whether this 97-kDa band I protein was also heat-inducible, clear lysates from *H. cutirubrum* grown at different temperatures (37, 42, and 52°C for 2–5 days) were applied to ATP-agarose columns equilibrated with buffer A containing 4 M NaCl. As clearly shown in Fig. 3A, the amount of band I protein bound to the ATP-agarose column increased with increases in the growth temperature. Some of the several faint bands with molecular masses of around 60 kDa were also heat-inducible, but another major band with a molecular mass of 24 kDa was not. Since we found that the affinity of band I protein to ATP-agarose was lower than those of other proteins, we partially purified band I protein by elution from the ATP-agarose column with buffer A containing 4 M NaCl and 0.2 mM ATP to more than 90% homogeneity (Fig. 3B). It was also found that Mg^{2+} ions were required for binding of band I protein to the ATP-agarose column (H. Tokunaga, unpublished result).

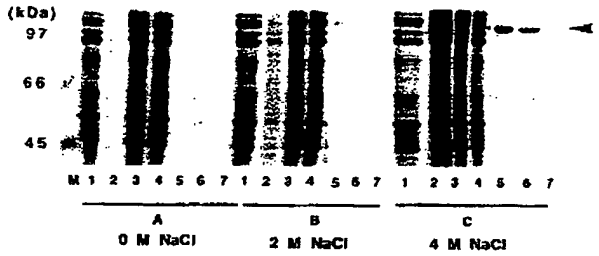

Fig. 2. SDS–PAGE analysis of eluents from ATP-agarose affinity column chromatography. *Halobacterium cutirubrum* cells were grown at 42°C and cell homogenates were prepared. The concentrations of NaCl in the buffers used for cell disruption and ATP-agarose column chromatography were 0 (A), 2(B), and 4 M (C). *Lane M*, low-molecular-mass standards (BioRad); *lane 1*, crude homogenates (20 μg of protein); *lane 2*, flowthrough fraction from the ATP-agarose column; *lanes 3* and *4*, first and second washes from the ATP-agarose column, respectively; *lanes 5–7*, first, second, and third eluted fractions from the ATP-agarose column, respectively. The arrow shows the position of 97-kDa protein.

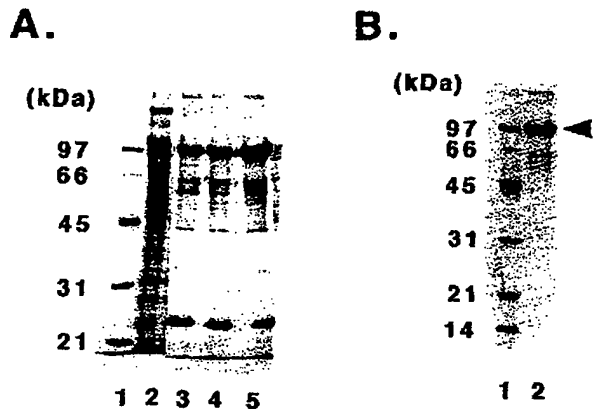

Fig. 3. (A) SDS–PAGE analysis of eluents from ATP-agarose affinity column chromatography with different growth temperatures. *Lane 1*, low-molecular-mass standards (BioRad); *lane 2*, crude homogenate (20 μg of protein); *lanes 3–5*, growth temperatures of cells were 37, 42, and 52°C, respectively. (B) SDS–polyacrylamide gel electrophoretogram of partially purified 97-kDa protein. *Lane 1*, low-molecular-mass standards (BioRad); *lane 2*, partially purified 97-kDa band I protein eluted from the ATP-agarose column with buffer A containing 4 M NaCl and 0.2 mM ATP. The arrow shows the position of 97-kDa protein.

An attempt to purify band I protein to homogeneity using several chromatographic techniques was not successful, as small amounts of contaminating proteins with apparent molecular masses of around 60–64 kDa cannot be removed completely.

3.2. Determination of NH_2-Terminal Amino Acid Sequence of Band I Protein

To identify the apparent 97-kDa band I protein, the preparation shown in Fig. 3B, lane 2, was subjected to SDS–PAGE, electroblotting onto a ProBlott membrane, and NH_2-terminal amino acid sequence analysis. The sequence was determined to be that shown at the top of Fig. 4. It was identical to the deduced NH_2-terminal amino acid sequence of the *dnaK* gene isolated from *H. cutirubrum*, the molecular weight of which was calculated to be 68,495, and showed high local similarity to the deduced NH_2-terminal amino acid sequences of hsp70 genes isolated from other archaebacteria (*H. marismortui* and *Methanosarcina mazei*), chloroplasts (pumpkin and garden pea), and prokaryotes (*Thiobacillus ferrooxidans*) using BLASTP or FASTA

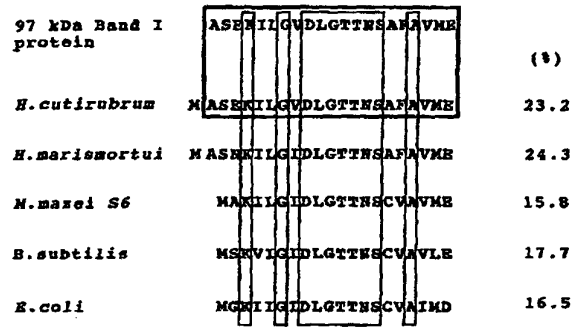

Fig. 4. Alignment of the NH_2-terminal sequences of band I protein from *Halobacterium cutirubrum* (this study) and those deduced from several Hsp70 genes. N-terminal sequences of Hsp70s from *Halobacterium marismortui* (Gupta and Singh, 1992), *Methanosarcina mazei S6* (Macario *et al.*, 1991), *Bacillus subtilis* (Wetzstein *et al.*, 1992), and *E. coli* (Bardwell and Craig, 1984) are shown in the middle column. The sequences in the thin and bold boxes were common to six and two species, respectively. The numbers indicate the percentage of acidic amino acid residues calculated from the deduced amino acid composition in the whole sequence.

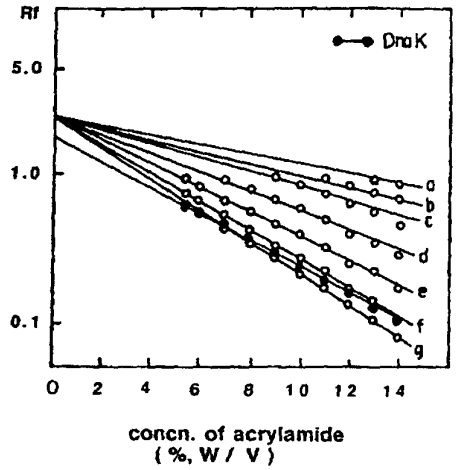

Fig. 5. Ferguson plots of 97-kDa protein. Proteins were subjected to SDS–PAGE according to the method of Laemmli at various concentrations of acrylamide. R_f (mobility of proteins relative to that of the dye front) was plotted on a log scale against acrylamide concentration. The reference proteins used were lysozyme (a), trypsin inhibitor (b), carbonic anhydrase (c), ovalbumin (d), serum albumin (e), phosphorylase B (f), and β-galactosidase (g).

software on GenomeNet. These sequence similarities strongly suggested that the 97-kDa band I protein was a gene product of *H. cutirubrum dnaK*, although the apparent (97 kDa) and calculated (68 kDa) molecular masses were different.

3.3. Ferguson Plot of Band I Protein

One possible reason for the discrepancy between the experimental and calculated molecular masses of *H. cutirubrum* DnaK is that the apparent molecular mass of 97 kDa on SDS–PAGE may not be accurate due to the possible aberrant mobility of band I protein on SDS–PAGE. To examine the behavior of band I protein on SDS–PAGE, we carried out SDS–PAGE using various concentrations (5.5–14%) of acrylamide, and plotted mobilities (R_f) of standard proteins (Bio-Rad 161-0304) and band I protein against the concentration of acrylamide (Ferguson plot, Fig. 5). Band I protein clearly showed aberrant behavior, indicating that SDS–PAGE was not suitable for determination of the correct molecular mass of this protein.

3.4. Determination of Internal Amino acid Sequences of Tryptic Peptides of Band I Protein in *H. cutirubrum*

Band I protein blotted onto a ProBlott membrane was digested by trypsin, extracted from the membrane, and subjected to reverse-phase HPLC to obtain internal peptides. We isolated nine peaks of peptides on HPLC, and their NH_2-terminal amino acid sequences were determined (Table I). Amino acid sequence analyses of three peaks at retention time $(Rt)_{31.0}$ min, $RT_{43.0}$ min and $RT_{60.1}$ min revealed that each peak yielded two phenylthiohydantoin (PTH)-amino acids in each cycle with equal recovery (Table I). This result indicated that these peaks contained two distinct peptides. These sequences could be aligned as shown in Table I with complete identity with the amino acid sequences deduced from the *H. cutirubrum dnaK* gene sequence. All amino acid sequences of other tryptic peptides coincided perfectly with the deduced amino acid sequence of *H. cutirubrum dnaK*. The amino acid residues shown in Table I (a total of 207 residues) covered 1/3 of the full-length amino acid sequence of DnaK. The deduced amino acid sequence of *dnaK* also indicated that the amino acid residues preceding the NH_2-terminus of each tryptic peptide were, as expected, Lys or Arg. These observations again strongly suggest that the apparent 97-kDa band I protein is DnaK from *H. cutirubrum*.

3.5. Expression of *H. cutirubrum* DnaK Homologue Gene

To confirm that the apparent 97-kDa band I protein is identical to the DnaK homologue from *H. cutirubrum*, we attempted to express *H. cutirubrum dnaK* gene in

Table I. Amino Acid Sequences of Internal Tryptic Peptides of Band I Protein[a]

Amino acid sequence	Residue number in DnaK (Total 629)	Retention time (RT)
ILGVDLGTTNSAFAVME	6–22	43.0
NQAVQNPDQTIASIKR	57–72	31.0
DAEEYLGQDVEK	104–115	30.3
AVITVP	116–121	38.7
DAGEIAGFDVER	134–145	34.1
KETTVNLPFVTATDSGPVHL	250–269	43.0
ATFESITEDLIER	276–288	44.7
SDIDDVILVGGSTR	305–318	37.1
NVNPDEAVALGAAVQGGVLS GEVDD	337–361	60.1
VFTTAADNQTSVQIR	398–412	31.0
EIRSENKLLGDFILTGIPPA PAGTD	419–443	60.1
AESLLEENEELVDEDLEADV NDAIDDVQAVLDEDEP	519–554	61.2

[a] Residue numbers in the second column are those of amino acid sequence deduced from *dnaK* gene. Number 1 represents the first methionine residue.

E. coli BL21 (DE3). The result, shown in Fig. 6, indicates that in *E. coli* BL21 (DE3) containing pET3a, no protein band around 97KDa was induced with and without IPTG induction (lane 3 and 2). On the other hand, a protein corresponding to the 97-kDa band I accumulated strongly in *E. coli* BL21 (DE3) containing pET3HcK under IPTG Induction (lane 5). The mobility of this accumulated protein (lane 5) on SDS–PAGE was the same as that of DnaK homologue from *H. cutirubrum* (lane 6).

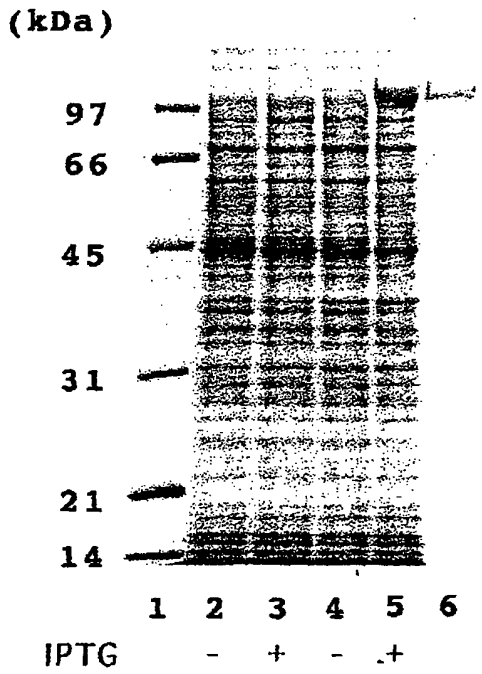

Fig. 6. Expression of *Halobacterium cutirubrum* DnaK homologue. *Escherichia coli* BL21 (DE3) cells which contain pET3a or pET3Hck were grown at 37°C to OD_{600} of approximately 0.8 and expression was induced with 0.2 mM IPTG for 2.5 hr. Total cell protein samples (20 μg) were run on 10% acrylamide gel followed by staining with Coomassie blue. *Lane 1*, low-molecular-mass standards (BioRad); *lanes 2 and 3*, *E. coli* BL21 (DE3) containing pET3a; *lanes 4 and 5*, *E. coli* BL21 (DE3) containing pET3HcK; *lanes 3 and 5*, cultures were induced with 0.2 mM IPTG; *lane 6*, 97-kDa protein purified from *Halobacterium cutirubrum*.

4. DISCUSSION

The halophilic archaebacteria live in extremely halophilic environments such as the Dead Sea and the Great Salt Lake, and maintain an intracellular osmotic balance against the environment by accumulating K^+ ions in their cytoplasm (Kushner, 1978). Therefore, the proteins from these microorganisms are expected to show properties distinct from those that function under normal osmotic pressure. Several proteins from these bacteria, such as polypeptide elongation factor Tu (Guinet *et al.*, 1988), β-galactosidase (Holmes *et al.*, 1997), protease (Kim and Dordick, 1997), seryl-tRNA synthetase (Taupin *et al.*, 1997), and glutamate dehydrogenase (Britton *et al.*, 1998), have been studied. Malate dehydrogenase from halopilic bacteria has been extensively investigated (Cendrin *et al.*, 1993; Madern and Zaccai, 1997), and its three-dimensional structure was also determined (Dym *et al.*, 1995). Our research interest lies in the synthesis and folding of proteins in extremophiles such as thermophiles and halophiles which grow under extreme environmental

conditions. We have identified some major Hsps in moderately halophilic eubacteria (Tokunaga *et al.*, 1997*a*), and purified and characterized a GroEL homologue from the moderately eubacterial halophile *Pseudomonas* species (Tokunaga *et. al.*, 1997*b*).

In the genetic study, it was reported that Hsps from archaebacteria were regulated at the transcriptional level (Kuo *et al.*, 1997). The first research focused on Hsps of extremely halophilic archaebacteria was that of Daniels *et al.* (1984). They found that a limited number of Hsps were induced upon heat shock, and the apparent molecular masses of Hsps on SDS–PAGE were classified into three narrow ranges, high (105,000–75,000 Da), intermediate (45,000–44,000 Da), and low (28,000–21,000 Da). However, the respective heat-inducible proteins from halophilic archaebacteria remained to be characterized. Here, we cultured *H. cutirubrum* at elevated temperatures, and found that the profile of accumulated proteins with increases in temperature was similar to that of Hsps of *H. trapanicium* and *H. marismortui* (Daniels *et al.*, 1984).

Hsp110 and Hsp90 families have been extensively investigated in eukaryotic cells, and in eukaryotic and prokaryotic cells, respectively (Lindquist and Craig, 1988). In *E. coli*, however, the majority of about 20 species of Hsps have apparent molecular masses of lower than around 70 kDa, with the exceptions of Hsp90, protease La (Niedhardt *et al.*, 1984) and clpB protein (Woo *et al.*, 1992), which have been extensively characterized. As shown in Fig. 1, we found three heat-inducible proteins in *H. cutirubrum* with apparent molecular masses larger than 70 kDa on SDS–PAGE, and speculated that they might be homologues of Hsp110 or Hsp 90 families. However, the NH_2-terminal and internal amino acid sequences revealed that the apparent 97-kDa band I protein (the slowest migrating band of these three) was most likely the gene product of the *dnaK* homologue from *H. cutirubrum* (Gupta and Singh, 1994). Gupta and Singh reported that only a single ~9-kb *Bam*HI band from *H. cutirubrum* chromosomal DNA hybridized with a 0.65-kb PCR-amplified *dnaK* probe, suggesting that there was no other gene closely related to *dnaK* in *H. cutirubrum*.

To resolve the discrepancy between the apparent molecular mass on SDS–PAGE and the calculated value from the nucleotide sequence of *dnaK*, we carried out SDS–PAGE with various concentrations of acrylamide, and examined the mobility of band I protein together with molecular weight standards (Ferguson plot). We found that the band I protein showed an anomalous mobility on SDS–PAGE (Fig. 5), and that the true molecular mass of band I protein could not be determined accurately by this method. In general, the proteins from extremely halophilic archaebacteria contain higher percentages of acidic amino acid residues than those from nonhalophilic organisms. We calculated the contents of acidic amino acid residues of several DnaK proteins (Fig. 4), and noticed that DnaK from the halophilic archaebacteria *H. cutirubrum* (23.2%) and *H. marismortui* (24.3%) contained significantly larger amounts of acidic amino acid residues than those from the nonhalophilic archaebacterium *M. mazei* (15.8%) and the eubacteria *E. coli* (16.5%) and *B. subtilis* (17.7%). This high content of negative charges might decrease the amount of bound SDS on DnaK, or might cause an unusually elongated conformation of the DnaK–SDS complex, resulting in an aberrant mobility observed on SDS–PAGE. In fact, Armstrong and Roman (1993) reported that the high content of acidic amino acid residues caused anomalous electrophoretic behavior of a virus protein. Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometric analysis to determine the accurate molecular mass of the band I sample with reduced salt concentration was not successful because of the appearance of protein aggregates under low salt concentration. The possibility that inefficient reduction of disulfide bonds may cause anomalous mobility of DnaK homologue on SDS–PAGE is ruled out because it contains no cysteine (Gupta and Singh, 1994).

To compare the mobility of *H. cutirubrum* DnaK homologue and 97-kDa protein SDS–PAGE, we expressed the *H. cutirubrum dnaK* gene in *E. coli* BL21 (DE3). As described in Section 3, we confirmed that the 97-kDa protein was identical to DnaK homologue in *H. cutirubrum*. In this study we reported that the 97-kDa protein which was a DnaK homologue could bind to the ATP-agarose column in the presence of 4 M NaCl. There are two possible mechanisms for the observed salt concentration dependence of the 97-kDa protein binding to ATP: This protein requires 4 M or higher salt concentration for the native conformation to bind ATP, or the binding constant depends on the salt concentration and the maximum binding is achieved at or above 4 M.

We identified and partially purified DnaK homologue, a major heat shock protein, from an extremely halophilic archaebacterium. Under extremely high intracellular salt conditions, interesting questions remain. How do halophilic proteins fold? How do molecular chaperones assist the protein folding process? Does the use of halophilic chaperones help *in vitro* refolding of halophilic proteins? The characterization of this unique chaperone will help in the investigation of refolding of extremely halophilic proteins.

REFERENCES

Armstrong, D. J., and Roman, A. (1993). *Biochem. Biophys. Res. Commun.* **192**, 1380–1387.

Bardwell, J. C. A., and Craig, E. A. (1984). *Proc. Natl. Acad. Sci. USA* **81**, 848–852.

Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994). *Nature* **371**, 578–586.

Britton, K. L., Stillman, T. J., Yip, K. S. P., Forterre, P., Engel, P. C., and Rice, D. W. (1998). *J. Biol. Chem.* **273**, 9023–9030.

Bukau, B., and Horwich, A. L. (1998). *Cell* **92**, 351–366.

Cendrin, F., Chroboczek, J., Zaccai, G., Eisenberg, H., and Mevarech, M. (1993). *Biochemistry* **32**, 4308–4313.

Daniels, C. J., McKee, A. H. Z., and Doolittle, W. F. (1984). *EMBO J.* **3**, 745–749.

Dym, O., Mevarech, M., and Sussman, J. L. (1995). *Science* **267**, 1344–1346.

Guinet, F., Frank, R., and Leberman, R. (1988). *Eur. J. Biochem.* **172**, 687–694.

Gupta, R. S., and Singh, B. (1992). *J. Bacteriol.* **174**, 4594–4605.

Gupta, R. S., and Singh, B. (1994). *Curr. Biol.* **4**, 1104–1114.

Haniu, M., Horan, T., Arakawa, T., Le, J., Katta, V., Hara, S., and Rohde, M. F. (1996). *Biochemistry* **35**, 13040–13046.

Hartl, F. U. (1996). *Nature* **381**, 571–580.

Holmes, M. L., Scopes, R. K., Moritz, R. L., Simpson, R. J., Englert, C., Pfeifer, F., and Dyall-Smith, M. L. (1997). *Biochim. Biophys. Acta* **1337**, 276–286.

Kim, J., and Dordick, J. S. (1997). *Biotech. Bioeng.* **55**, 471–479.

Kuo, Y.-P., Thompson, D. K., Jean, A. S. T., Charlebois, R. L., and Daniels, C. J. (1997). *J. Bacteriol.* **179**, 6318–6324.

Kushner, D. J. (1978). In *Microbial Life in Extreme Environments: Life in High Salt and Solute Concentrations, Halophilic Bacteria* (Kushner, D. J., Ed), Academic Press, London, pp. 317–368.

Laemmli, U. K. (1970). *Nature* **227**, 680–685.

Lindquist, S., and Craig, E. A. (1988). *Annu. Rev. Genet.* **22**, 631–677.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265–275.

Macario, A. J. L., Dugan, C. B., and Conway de Macario, E. (1991). *Gene* **108**, 133–137.

Madern, D., and Zaccai, G. (1997). *Eur. J. Biochem.* **249**, 607–611.

Mevarech, M., Hirsch-Twizer, S., Goldman, S., Yakobson, E., Eisenberg, H., and Dennis, P. P. (1989). *J. Bacteriol.* **171**, 3479–3485.

Niedhardt, F. C., VanBogelen, R. A., and Vaughn, V. (1984). *Annu. Rev. Genet.* **18**, 295–329.

Sehgal, S. N., and Gibbons, N. E. (1960). *Can. J. Microbiol.* **6**, 165–169.

Taupin, C. M. J., Hartlein, M., and Leberman, R. (1997). *Eur. J. Biochem.* **243**, 141–150.

Tokunaga, M., Matsuoka, K., and Tokunaga, H. (1997*a*). *Biosci. Biotech. Biochem.* **61**, 1388–1390.

Tokunaga, M., Miyawaki, H., Shiraishi, Y., and Tokunaga, H. (1997*b*). *FEMS Microbiol. Lett.* **152**, 321–326.

Wetzstein, M., Voelker, U., Dedio, J., Loebau, S., Zuber, U., Schiesswohl, M., Herget, C., Hecker, M., and Schumann, W. (1992). *J. Bacteriol.* **174**, 3300–3310.

Woo, K. M., Kim, K. I., Goldberg, A. L., Ha, D. B., and Chung, C. H. (1992). *J. Biol. Chem.* **267**, 20429–20434.

Xu, Z., Horwich, A. L., and Sigler, P. B. (1997). *Nature* **388**, 741–750.

Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. E., and Hendrickson, W. A. (1996). *Science* **272**, 1606–1614.

JOURNAL OF PROTEIN CHEMISTRY

Volume 18, Number 8 **November 1999**

CONTENTS

JOURNAL OF PROTEIN CHEMISTRY

Journal of Protein Chemistry is an international journal devoted to the publication and communication of original research work in all aspects of investigations concerned with the elucidation of the molecular basis for protein function. The journal places equal emphasis on all facets of protein activity (e.g., enzymes, hormones, protein antigens, antibodies, receptors, etc.). Since approaches to function are meaningful only with detailed knowledge of structure and are equally based on innovations of applications from a diversity of organic and physical chemistry approaches, the journal also publishes original articles dealing with protein structure (covalent or three-dimensional), reactions of functional groups, protein conformation, and peptide synthesis. To ensure maximum effectiveness and full accessibility to all investigators, the journal does not levy a page charge and emphasizes rapid publication of accepted manuscripts. Reports may be submitted as either regular papers or brief communications. All manuscripts are subject to review by referees who are experts in their fields. The referees' recommendations determine the acceptability of submitted manuscripts. The majority of reports are published as regular papers. A regular paper constitutes a complex piece of work that contributes significantly to the understanding of a particular problem. The work should be described concisely but in sufficient detail to permit experimental confirmation by other interested investigators. Brief communications constitute manuscripts based on observations that are considered timely and, accordingly, their rapid dissemination will be beneficial to the advancement of the field. These manuscripts should be no more than four printed pages and are assured publication in the next issue after acceptance. They are judged by the referees on the basis of not only scientific merit, but also fulfillment of the requirement for urgency.

Journal of Protein Chemistry is published monthly except March, June, September, and December by Kluwer Academic/Plenum Publishers, 233 Spring Street, New York, N.Y. 10013-1578. *Journal of Protein Chemistry* is abstracted or indexed in Chemical Abstracts, Current Awareness in Biological Sciences, Current Contents, Excerpta Medica, and Index Medicus.

Journal of Protein Chemistry participates in the Copyright Clearance Center (CCC) Transactional Reporting Service. The appearance of a code line at the bottom of the first page of an article in this journal indicates the copyright owner's consent that copies of the article may be made for personal or internal use. However, this consent is given on the condition that the copier pay the flat fee of $16.00 per copy per article (no additional per-page fees) directly to the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, Massachusetts 01923, for all copying not explicitly permitted by Sections 107 or 108 of the U.S. Copyright Law. The CCC is a nonprofit clearinghouse for the payment of photocopying fees by libraries and other users registered with the CCC. Therefore, this consent does not extend to other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creating new collective works, or resale, nor to the reprinting of figures, tables, and text excerpts. 0277-8033/99 $16.00

Advertising inquiries should be addressed to Advertising Sales, Kluwer Academic Publishers, P.O. Box 989, 3300 AZ Dordrecht, The Netherlands—telephone 31 78 639 2278 and fax 31 78 639 2323.

Subscription inquiries and subscription orders should be addressed to the publisher at Subscription Department, Kluwer Academic/Plenum Publishers, 233 Spring Street, New York, N.Y. 10013-1578 or faxed to the Subscription Department at its number (212) 807-1047, or may be telephoned to the Subscription Department's Journal Customer Service at (212) 620-8035, -8404, -8466, or -8472, or -8490. Subscription rates:

Volume 18, 1999 (8 issues) $575.00 (outside the U.S., $675.00). Price for individual subscribers certifying that the journal is for their personal use, $75.00 (outside the U.S., $88.00).

Volume 19, 2000 (8 issues) — Traditional **print** subscription: $645.00 (outside the U.S., $755.00). Price for individual subscribers certifying that the journal is for their personal use, $83.00 (outside the U.S., $97.00). **Electronic** subscription: $645.00 (outside the U.S., $755.00). **Combination** print and electronic subscription: $774.00 (outside the U.S., $906.00).

Postmaster: Send address changes to *Journal of Protein Chemistry,* Kluwer Academic/Plenum Publishers, 233 Spring Street, New York, N.Y. 10013-1578.

Printed in the USA.